Respectfully submitted,

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MARKED-UP VERSION OF SPECIFICATION:

paragraph on page 5, complete page to page 6, line 4:

β_2-agonists and glucocorticoids are the two most effective treatments available for asthma therapy and frequently are used in combination. Glucocorticoids are used principally because of their anti-inflammatory properties,[7] although additional beneficial effects of glucocorticoids in the asthmatic lung have been observed.[135] Synthetic glucocorticoids are efficacious for treating asthma and other diseases with associated inflammatory processes because they mimic glucocorticoids produced endogenously by the adrenal cortex. The cellular and molecular mechanisms of action of the glucocorticoids have been extensively studied.[135, 154] The current model of glucocorticoid action postulates intracellular glucocorticoid receptors, which in the absence of ligand, are complexed with heat shock proteins (hsp90, hsp56, hsp70 and an acidic 23 kD protein) (See Figure 3). Glucocorticoids are found in the blood bound to transcortin, albumin, and other serum proteins. Free glucocorticoids enter target cells, by still unidentified mechanisms[49, 152] and bind to glucocorticoid receptors causing dissociation of the associated heat shock proteins. Association of hsp90 with glucocorticoid receptors appears to maintain the hormone binding domain in its high affinity conformation.[113] The functional roles of the other associated heat shock proteins are not as well understood, but may include trafficking of the receptor within the cell.[113] The glucocorticoid receptor is a member of a superfamily of transcriptional regulators that include receptors for estrogens, progesterones, androgens, vitamin D, thyroid hormones, and retinoic acid.[83] Members of the superfamily share a similar structure with functional domains for binding of hormone, binding to DNA, and transcriptional activation. Hormone-receptor complex translocates from the cytoplasm to the nucleus. Activated glucocorticoid receptor with hormone bound has an increased affinity for binding to specific DNA sites termed glucocorticoid response elements (GRE) found within glucocorticoid reponsive genes. GREs can either be simple or composite.[48] Most simple GREs consist of two half-site hexamers separated by three nucleotides with resemblance to the consensus sequence GTCACAnnnTGTTCT **(SEQ ID NO:1)**. Association of glucocorticoid receptor, typically as a homodimer, to simple GREs results in enhanced transcription of the target gene. A second type of DNA sequence that binds glucocorticoid receptors, termed composite GREs, has been found in certain glucocorticoid-responsive genes.[31] At composite GREs, the hormone receptor complex interacts with both specific DNA sequences and other transcription factors to regulate transcription.[31, 47, 91] The first demonstrated composite GRE was shown to have binding sites for both the glucocorticoid receptor and activating protein-1 (AP-1).[31] AP-1 is a dimer of the oncogene

products *c-fos* and *c-jun*. Since glucocorticoid receptors are expressed in many cell types, composite GREs may explain how signal specificity can be achieved in a system with an apparent common final pathway.[48]

PAGE 13, 6TH FULL PARAGRAPH:

Figure 6 depicts the genomic structure of recombinant AAV vectors. For reference, the genomic structure of wildtype AAV is shown at the top. Descriptions of each vector can be found in the text. β_2AR(tag) refers to a cassette that contains the β_2AR coding region with an epitope (YPYDVPDYA, SEQ ID NO:2) added at the amino terminus of the receptor open reading frame. The epitope tag does not alter β_2AR function[147] and can be detected with a specific antibody.[100]

PAGE 14, 1st FULL PARAGRAPH:

Figure 8 depicts putative glucocorticoid response elements (GRE) in the rat β_2-AR gene. Figure 8A provides a schematic representation of the β_2AR gene. GREs are number and approximate locations are shown. Figure 8B shows the exact locations (+1 is the start of transcription) of the putative GREs. The third column shows the nucleotide sequence of each GRE (SEQ ID NOS:3-9) compared to the MMTV consensus GRE (SEQ ID NO:10). Underlined nucleotides match consensus. The number of matching mucleotides compared to the consensus GRE are shown in column 4.

PAGE 29, 6TH FULL PARAGRAPH:

Because SPOC1 cells express a wild-type β_2AR, it is useful to have a method to detect the expression of recombinant β_2AR in clonal lines infected with recombinant AAV. To accomplish this, an epitope-tagged β_2AR is used. The cDNA encoding the rat β_2AR are modified by insertion of the sequence encoding YPYDVPDYA (SEQ ID NOS:11-15) at the amino terminus of the receptor by oligonucleotide-directed mutagenesis. This modification has been performed on the human β_2AR and has been shown to not alter expression or function of the receptor.[147] This nine amino-acid epitope is recognized by the antibody 12CA5.[100] Thus, immunoblot analysis of membrane fractions prepared from SPOC1 cells can be used to detect recombinant receptor. Membrane fractions from infected SPOC1 cells are resolved on 10% SDS polyacrylamide gels, transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH). Immunoblotting is performed in 5% nonfat dry milk containing 2% Nonidet P-40 as previously described using primary antiserum at 1/600 and horseradish peroxidase-conjugated second antibody.[147] The

presence of recombinant β_2AR in clonal cell lines infected with recombinant AAV vector was detected, whereas mock-infected cells did not express the epitope-tagged β_2AR.

PAGE 43, LINE 12 TO PAGE 44, LINE 10, FULL PARAGRAPH:

First, corticosteroids are frequently used to treat asthmatic patients. This is done principally to control the inflammatory component of asthma. Therefore, expression of the transgene can be controlled by a therapeutic agent that most asthmatic patients already use. Second, glucocorticoids increase the rate of transcription of several genes including the β_2AR.[5] This aspect of glucocorticoid action is considered in the design of the optimal β_2AR transgene for functional testing in airway epithelial cells *in vitro* and *in vivo*. Classically, glucocorticoids exert their effects by binding to a cytoplasmic glucocorticoid receptor causing the release of an associated 90 kDa heat shock protein and thereby allowing translocation of the receptor to the nucleus. Within the nucleus, glucocorticoid receptors form dimers that bind to DNA within steroid-responsive genes at consensus sequences called glucocorticoid response elements. This interaction changes the rate of transcription of the gene, most often resulting in induction of transcription, but in some cases gene expression can be repressed. The present inventors have identified the core GRE in the rat β_2AR gene as it functions in the HepG2 cell line as discussed below. Based on this work and other evidence, the expression of the rat β_2AR gene is induced by glucocorticoids. In these studies, the SPOC1 cell line is used to functionally characterize the *cis*-acting elements in the β_2AR gene that are involved in glucocorticoid induction. Glucocorticoid receptors bind to the consensus sequence GGTACAnnnTGTTCT **(SEQ ID NO:10)**(where n is any nucleotide). In some instances this may be a straight-forward interaction in which the receptor dimer bound to the GRE then interacts with basal transcription factors[67] or other DNA-binding proteins[126,127] resulting in enhanced transcription of the target gene. However, in many cases the interactions are more complex. At composite GREs, the hormone receptor complex interacts with both specific DNA sequences and other transcription factors to regulate transcription of the target gene.[31,47,91] Some transcription factor binding elements that interact with glucocorticoid response elements include those for activating protein-1,[31] C/EBP[56] and hepatic nuclear factor 3 (HNF3).[148] Widely spaced glucocorticoid response elements have been shown to function in tandem to induce expression of the tryptophan oxygenase gene.[27] The data obtained from transient expression of β_2AR-luciferase fusion genes in HepG2 cells indicates complex regulation of β_2AR gene expression by glucocorticoids that appears to involve other as yet unidentified genetic elements.

PAGE 46, LINE 30 TO PAGE 47, LINE 9, FULL PARAGRAPH:

To test further the involvement of GRE_5 in glucocorticoid regulation of β_2AR expression, a plasmid pβ_2ARm1(-3129/+126) was constructed that had been mutated at position +6 of GRE_5 (GGGTGAGCTGTTCT → GGGTGAGCTATTCT, **SEQ ID NOS:16 AND 17**). This mutation, the same base change in oligonucleotide m1GRE_5 (Figure 10), is essential for glucocorticoid inducibility of a MMTV GRE.[103] The results demonstrate loss of glucocorticoid inducibility using pβ_2ARm1(-3129/+126) (Figure 11). Interestingly, in the absence of added dexamethasone, activity of pβ_2ARm1(-3129/+126) was markedly lower than that of pβ_2AR(-3129/+126) (Figure 11). It appears that basal expression of pβ_2AR(-3129/+126) in HepG2 cells that are over-expressing glucocorticoid receptor is relatively high despite removal of glucocorticoids from serum by charcoal stripping. Forty-eight hours prior to transfection, the HepG2 cells are switched to charcoal-stripped serum. Alternatively, GRE_5 contributes to basal activity of the β_2AR gene promoter.